Structure of an Optically Active Allene-containing Tetraester Triglyceride Isolated from the Seed Oil of *Sapium sebiferum**

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ABSTRACT: The optically active lipid isolated from the seed oil of the Chinese tallow tree, *Sapium sebiferum*, previously considered to be a triglyceride containing 2,4-decadienoic acid esterified on one of the primary hydroxyls of glycerol, is shown to be tetraester triglycerides (I). Mass spectral analysis showed that in the completely hydrogenated I stearic acid is esterified to one primary and the secondary hydroxyl group of glycerol. The other primary hydroxyl of glycerol is esterified to 8-hydroxyoctanoic acid, and its ω-hydroxyl group, in turn, is esterified to decanoic acid. In I the predominant common fatty acids are linoleic and linolenic acids. The eight-carbon hydroxy acid is 8-hydroxy-5,6-octadienoic acid (II). Three derivatives of compound II were prepared and were found to be optically active at 580 mμ and showed increased rotation as the ultraviolet region was approached. The ten-carbon moiety in compound I is the carboxyl conjugated acid, 2,4-decadienoic acid (III). The optical activity observed in compound I is probably due to the allene function rather than to an asymmetric β-carbon of glycerol. From mass spectral studies of the components of compound I, some insight was gained about the distribution pattern of the long-chain acids on the primary and secondary hydroxyls of glycerol. The structure of compound I was deduced from infrared, near-infrared, n.m.r., and mass spectra, and from chemical evidence. The methyl ester acetate of compound II, 2,3-octadiene-1,8-diol diacetate, and the methyl ester of the ester of compounds II and III exhibited optical rotations, $[\alpha]_D^{20}$, of -40, -38, and $-46°$, respectively.

In a study of the seed oil of the Chinese tallow tree, *Sapium sebiferum*, Huang *et al.* (1949) noted that the oil had a specific rotation of $[\alpha]_D^{20}$ $-5.0°$. The oil had an ultraviolet spectrum with the absorption maximum occurring at 260 mμ. In an investigation of the same oil Crossley and Hilditch (1949) demonstrated that the carboxyl conjugated acid, 2,4-decadienoic acid, was the chromophore responsible for the ultraviolet absorption of the oil.

In a recent report from our laboratory a procedure was described for isolating the optically active lipid from the total oil (Maier and Holman, 1964). The optically active component was about 26% of the total oil and had a specific rotation of $[\alpha]_D^{20}$ $-21.9°$. Gas-liquid chromatography showed it to be the only lipid fraction containing 2,4-decadienoic acid. Countercurrent fractionation of the active lipid yielded four fractions, all of which rotated light at the sodium line to about the same extent. In agreement with the findings of Hirayama (1961), it was noted that about one-third of the fatty acids in the optically active lipid as well as in its four countercurrent fractions were 2,4-decadienoic acid. These and other observations suggested that the optically active lipid was a triglyceride in which 2,4-decadienoic acid was esterified solely to a primary hydroxyl of the glycerol moiety. Two other long-chain fatty acids, predominately linoleic and linolenic, were esterified to the other two hydroxyls of glycerol. It was considered that the close proximity of the 2,4-decadienoic acid to the potentially asymmetric β-carbon of glycerol provided a degree of asymmetry to the molecule sufficient to account for the observed optical rotation. We have now been able to show, largely through mass spectral studies, that the structure is considerably more complex.

Experimental

The methods used for the isolation, fractionation, and countercurrent distribution of the oil have been described previously (Maier and Holman, 1964). For fatty acid analysis of the glycerides by gas-liquid chromatography, methyl esters of the fatty acids were prepared by interesterification with 5% HCl in anhydrous methanol. Another useful analytical technique involved treating the lipid with $LiAlH_4$ and then acetylating the alcohols with acetic anhydride (Horrocks and Cornwell, 1962).

To accumulate the alcohol acetates in sufficient amounts for characterization, 200–400 mg were

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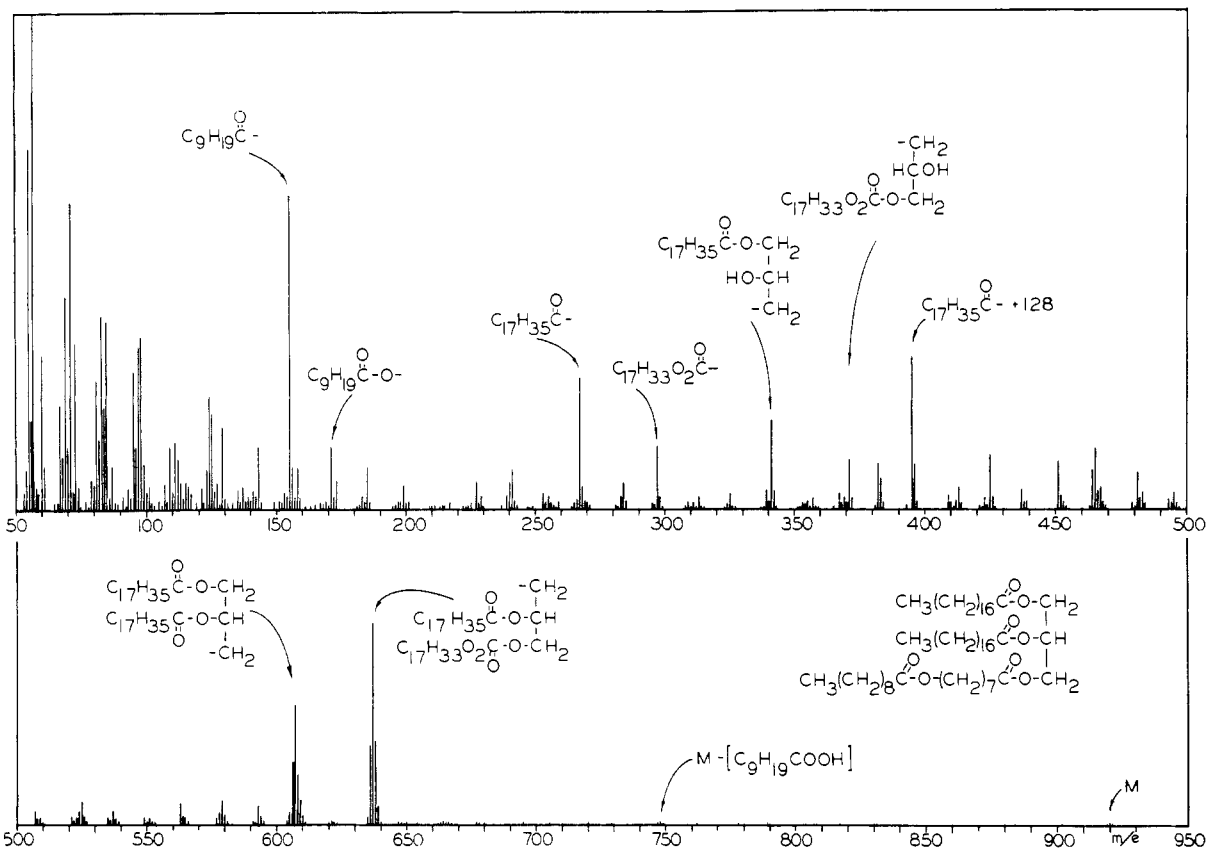


FIGURE 1: Mass spectrum of hydrogenated optically active triglyceride from *Sapium sebiferum*.

chromatographed on a column (1 × 40 cm) packed with silica gel G (W. R. Grace Co.) and cooled with a water jacket. The column was eluted with increasing concentration of diethyl ether in petroleum hydrocarbon (bp 34–45°). The elution was monitored by thin-layer chromatography using the solvent system petroleum hydrocarbon–diethyl ether–acetic acid (80:20:2). Gas-liquid chromatography was also used to monitor the column.

A Barber-Colman Model 10 instrument equipped with an argon ionization detector was used for the chromatography of both the methyl esters and alcohol acetates. The glass column, 2.5 m × 0.65 cm, was packed with 20% ethylene glycol succinate polyester on Gas Chrom P (Applied Science Laboratories). The chromatograph was run at 160° with an argon flow rate of 60 ml/min.

Ozonolysis and reduction was carried out as described by Privett and Nickell (1962). In this procedure the ozonides are reduced with Lindlar's catalyst to the aldehydes and then separated by gas-liquid chromatography.

Optical rotatory dispersion measurements were made with a Rudolph recording spectropolarimeter with a mercury discharge lamp as the energy source. All points were recorded manually and, when necessary, suitable correction factors were used as determined by the rotational spectrum of quartz. The measurements were made in a 5.0-cm cell with dichloromethane as solvent. With the samples and solvents used in this study, the limit of the instrument was found to be 339 mμ.

Infrared spectra were run in carbon tetrachloride with a Beckman IR-8 grating instrument. The cells had a light path of 0.1 mm and were made of sodium chloride. Near-infrared spectra were run in carbon tetrachloride using a Beckman DK-2 spectrophotometer. The n.m.r. spectrum was determined using a Varian A60 nuclear magnetic resonance spectrometer and tetramethylsilane as reference.

All of the mass spectral studies were carried out with an A.E.I. MS-9 double-focusing mass spectrometer. The samples were introduced into the mass spectrometer in a detachable cup at the end of a ceramic rod. The probe was heated to about 200° to provide sufficient vapor pressure from the triglyceride samples.

Results

Gas-liquid chromatography analysis of the methyl esters from the native optically active lipid showed that about 95 mole % of the acids are composed of 2,4-decadienoic acid and common eighteen-carbon straight-chain acids. According to our original proposal, the hydrogenated lipid should therefore have been primarily a diacid triglyceride (C_{10}, C_{18}, C_{18}) having 49 carbon atoms and a molecular weight of 778. However, the

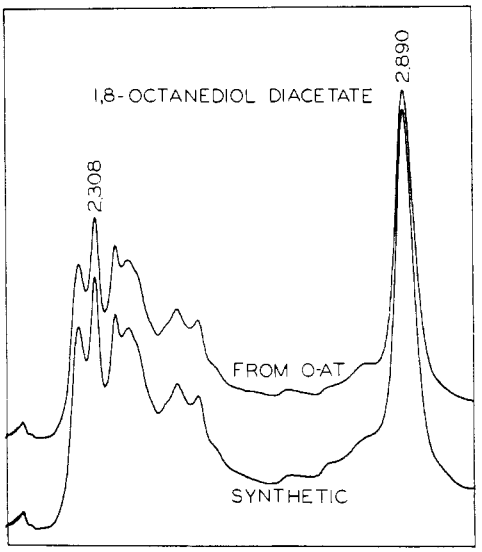


FIGURE 2: Near-infrared spectra of isolated and synthetic octane-1,8-diol diacetates. O-AT signifies optically active triglyceride.

parent peak in the mass spectrum of the hydrogenated triglyceride, as shown in Figure 1, occurs at *m/e* 920, or 142 units higher than expected from our former proposal. Thus, a fragment which had escaped detection in our previous analyses corresponds to an eight-carbon hydroxy acid.

The complete structure of the saturated triglyceride given in Figure 1 can be directly deduced from the mass spectrum. Principal peaks are found for each acyl fragment cleaved from the molecule. The acyl group derived from decanoic acid occurs at *m/e* 155; at *m/e* 297 the intact eight- plus ten-carbon acyl fragment occurs; and at 267 the acyl group originating from stearic acid is found. In mass spectra of triglycerides (Barber *et al.*, 1964) fragments are observed which have mass numbers 74 and 128 units higher than each acyl group which is split directly from the glycerol. The peaks at *m/e* 341 and 395 correspond to the fragments 74 and 128 mass numbers higher than the acyl group derived from stearic acid. The peaks at *m/e* 371 and 425 are, respectively, 74 and 128 mass units greater than the intact C_8 plus C_{10} acyl group. The absence of peaks 74 and 128 units higher than *m/e* 155 shows that decanoic acid is not esterified to the glycerol moiety. The two peaks at *m/e* 637 and 607 correspond to the fragments remaining after acyloxy loss of stearic acid and the eighteen-carbon diester moiety, respectively. The $C_8 + C_{10}$ group is esterified to a primary hydroxy group of glycerol because a peak is found 14 mass numbers lower at 593. This fragment is formed by loss of $C_{17}H_{33}O_2COOCH_2$ from the molecule.

The eight-carbon fragment which had escaped detection prior to the examination of the mass spectrum was then isolated as several derivatives to further establish its structure. It was first isolated as the 1,8-octanediol diacetate by treating the saturated tetraester triglyceride

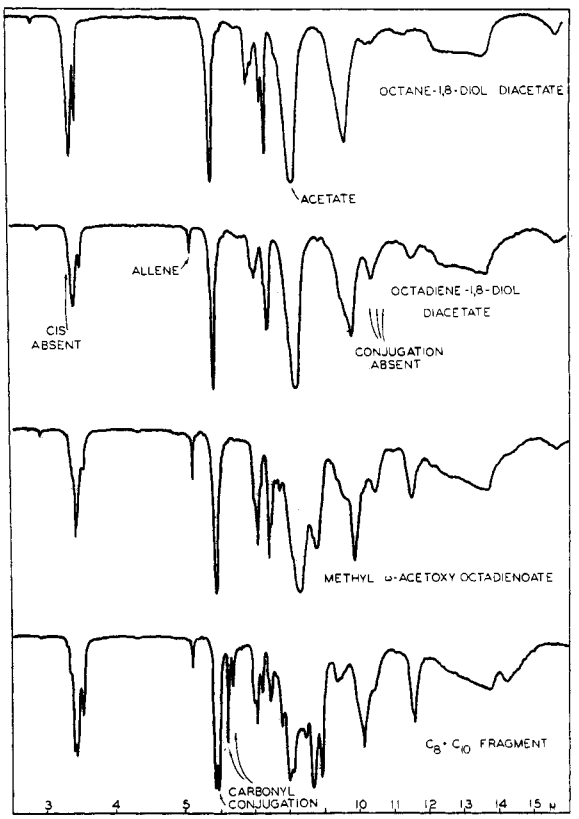


FIGURE 3: Infrared spectra of derivatives of the eight-carbon fatty acid moiety.

with $LiAlH_4$ followed by acetylation. The products of this reaction were separated by silica gel column chromatography. Figure 2 shows the near infrared spectrum of the isolated component along with that of authentic 1,8-octanediol diacetate. The first infrared spectrum in Figure 3 is that of the isolated 1,8-octanediol diacetate. This spectrum was also superimposable on that of the authentic compound.

When the native unsaturated tetraester triglyceride was treated with $LiAlH_4$ and acetylated in the same manner, the resulting eight-carbon diacetate had a retention time 1.57 times longer on gas-liquid chromatography than 1,8-octanediol diacetate, indicating unsaturation. The infrared spectrum of this compound is the second shown in Figure 3. The absence of absorption bands at 3.3 and 10.28 μ indicates that there are no isolated *cis* or *trans* double bonds. The ultraviolet spectrum indicated the absence of conjugated diene, triene, or carbonyl-conjugated double bonds. The only unsaturation in the molecule is due to an allene function which absorbs at about 5.1 μ (Wotiz *et al.*, 1952). Hydrogenation of the allene-containing compound yielded a component identical with 1,8-octanediol diacetate in gas-liquid chromatographic analysis.

Allene-containing 1,8-octadienol can exist in only two positional isomers in which the double bonds are in the 2,3 or 3,4 positions. To distinguish these, the nuclear magnetic resonance spectrum was obtained using a

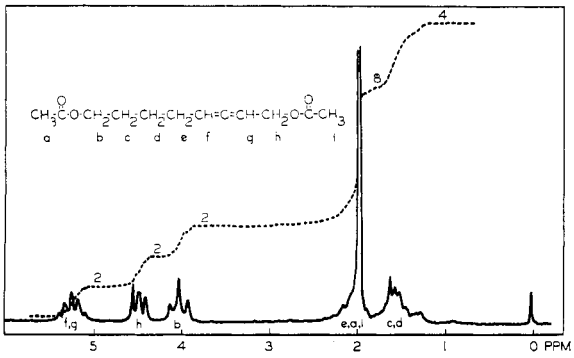


FIGURE 4: Nuclear magnetic resonance spectrum of octadiene-1,8-diol diacetate isolated from optically active triglycerides, and the proton assignments from which the structure is deduced.

Varian A60 nuclear magnetic resonance spectrometer. Proton assignments were made after comparison with published data (Jackman, 1959; Bhacca *et al.*, 1962) and are given in Figure 4. From the spectrum, as depicted in Figure 4, it was concluded that the structure was 2,3-octadiene-1,8-diol diacetate (equivalent to 5,6-octadiene-1,8-diol diacetate). The terminal allene double bond in this molecule is, therefore, located allylic to the hydroxyl group. Because in the $LiAlH_4$ treatment esters were reduced to alcohols, the possibility remained that in the native lipid the terminal allene double bond could have been conjugated with the ester carbonyl, although specific ultraviolet and infrared absorptions might be expected from such conjugation.

To isolate the eight-carbon fragment in another form, the unsaturated triglyceride was interesterified with methanolic HCl. Hydroxyl groups were then acetylated with acetyl chloride and the methyl ω-acetoxyoctadienoate was isolated using a silica gel column. Its infrared spectrum is the third in Figure 3. The absence of carbonyl conjugation at 5.7–6.5 μ indicated that the allene was located allylic to the hydroxy group. The compound was ozonized and the reduced ozonides yielded a substance which, upon gas-liquid chromatographic analysis, had the same retention time as the five-carbon aldehydo methyl ester obtained from the ozonolysis of methyl arachidonate, proving conclusively that the compound was methyl 8-acetoxy-5,6-octadienoate. Its ultraviolet spectrum showed no characteristic maxima in the region from 340 to 220 mμ. A mass spectrum of this substance yielded principal fragments compatible with this structure.

In addition to the methyl 8-acetoxy-5,6-octadienoate, a second allene-containing component was isolated from the same reaction mixture from which the former was

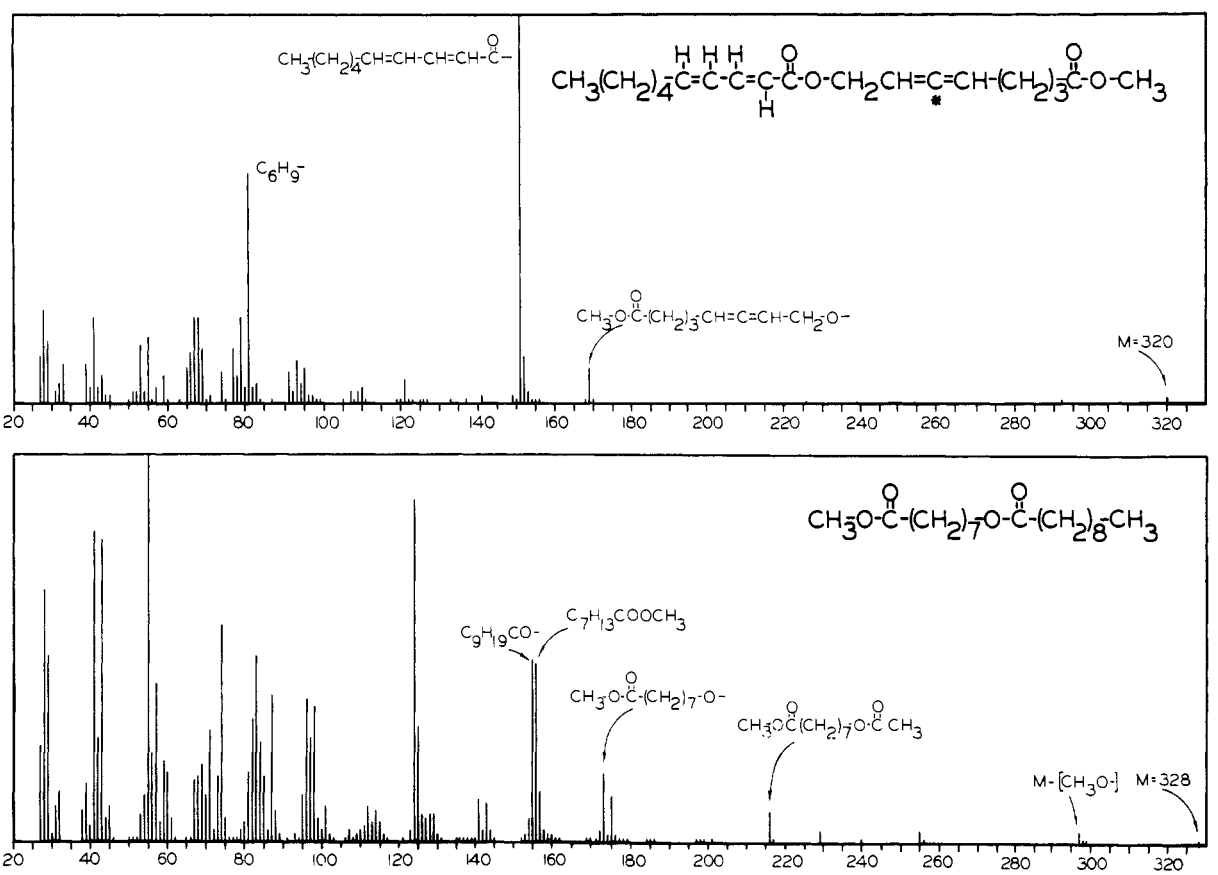


FIGURE 5: Mass spectrum of the unsaturated and the hydrogenated linear diester moiety.

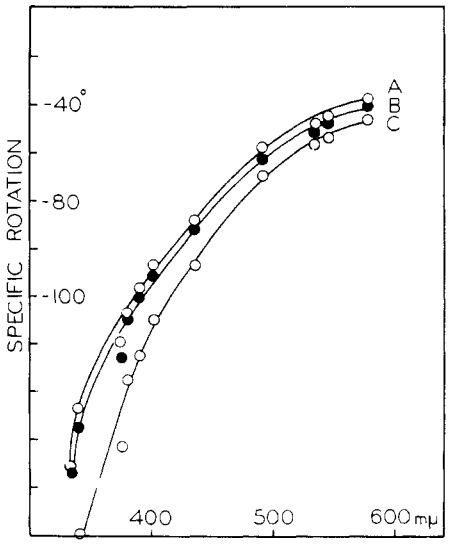


FIGURE 6: Optical rotatory dispersion curves of 2,3-octadiene-1,8-diol diacetate (A), methyl 8-acetoxy-5,6-octadienoate (B), and linear diester moiety (C). The solvent was dichloromethane.

isolated. The fourth infrared spectrum in Figure 3 shows that, in addition to strong allene absorption at 5.1 μ, the compound had split carbonyl absorption at 5.87 and 5.78 μ, indicating both conjugated and nonconjugated ester linkages. A compound of this nature could arise by incomplete interesterification of the native triglyceride, thereby yielding the methyl ester of the intact eight- plus ten-carbon fragment. The compound had an ultraviolet spectrum with a strong, smooth absorption maximum at 266 mμ, comparable to that of methyl 2,4-decadienoate. Conclusive proof of structure was provided by mass spectral analysis as shown in Figure 5. The parent ion peaks of the unsaturated and hydrogenated C_8 plus C_{10} esters occur, respectively, at 320 and 328 mass numbers, agreeing with the calculated values. Other principal fragments are appropriately labeled. The mass spectra confirm that these substances are the esters of eight-carbon hydroxy acids and ten-carbon fatty acids and agree with the structure deduced by the mass spectrum of the hydrogenated optically active triglyceride.

The allene function bestows permolecular symmetry upon a molecule and thus possible optical activity. The optical rotatory dispersion curves of the isolated allene compound in three forms in the range from 580 to 339 mμ are shown in Figure 6. All three components were active at 580 mμ and displayed increasingly negative optical activity as the ultraviolet region was approached.

As previously described, countercurrent fractionation of the optically active lipid yielded four fractions (Maier and Holman, 1964). A cursory examination indicated that the only difference among the fractions was in the long-chain fatty acid composition. As depicted in Figure 7, the native optically active triglyceride and four countercurrent fractions have the same infrared spectrum. The allene function is present in all fractions as evidenced by the small absorption band at 5.1 mμ. The last infrared spectrum is that of methyl 2,4-decadienoate, which agrees with the spectrum published by Crombie (1955), who established that the dienoic acid of the oil of *Sapium sebiferum* has the *trans*-2,*cis*-4 configuration. In the spectrum of the hydrogenated triglycerides, all evidence of unsaturation is absent, and the spectrum is that of a typical saturated triglyceride.

Figure 8 shows that the four countercurrent fractions rotate light to about the same extent as the natural mixture of optically active triglycerides. In addition to the allene function, these contain another potential asymmetric center in the β-carbon of glycerol. Since one primary hydroxyl of glycerol is esterified to the diester moiety, and the other primary position is linked in the normal manner, the β-carbon atom of glycerol may contribute to the observed rotation. If this were true, the hydrogenated glyceride should still rotate light. However, as shown in Figure 8, the hydrogenated lipid is inactive from 580 down to 250 mμ. Absence of measurable optical activity does not exclude the possibility of an asymmetric β-carbon atom of glycerol, for saturated asymmetric triglycerides have been observed to have no measurable rotation (Schlenk, 1962).

Recently, Barber *et al.* (1964) have shown that mass spectral analysis can be used for elucidating the complete structure of triglycerides. The structure of a synthetic triglyceride containing three different acids was determined directly from the fragmentation pattern. In an attempt to gain insight into the fatty acid distribution of the optically active triglyceride, spectra of the four countercurrent fractions were run. Figure 9 depicts the mass spectrum of the major component obtained, fraction A. Gas-liquid chromatographic analysis of this component showed that oleic, linoleic, and linolenic acids are the principal long-chain acids occurring in the ratio 12.6:25.3:16.2. The principal parent peak of this sample occurs at *m/e* 902, or 18 mass units lower than the hydrogenated optically active triglyceride. Since four of the double bonds are in the diester moiety, the major component must contain one linoleic and one linolenic acid moiety. The fragmentation pattern is characterized by the ease with which the conjugated ten-carbon fragment splits off from the parent. The peaks at *m/e* 734 and 751 arise by acyloxy and acyl cleavage, respectively, of the ten-carbon fragment from the parent. The fragment formed by loss of the diester moiety from the parent molecule is evident at 592. The principal or base peak at *m/e* 151 is the ten-carbon fragment itself. There are three peaks at 455–460 which correspond to the loss of the ten-carbon fragment, via acyloxy cleavage, plus the loss of one other fatty acid fragment. At 455, 457, and 459 mass numbers linolenic, linoleic, and oleic acids, respectively, are part of the observed fragment. Further information concerning the fatty acid composition can be obtained from the 261–265 mass region. At mass numbers 261, 263, and 265, respectively, the acyl ions derived from linolenic, linoleic, and oleic acids are found. Sixteen

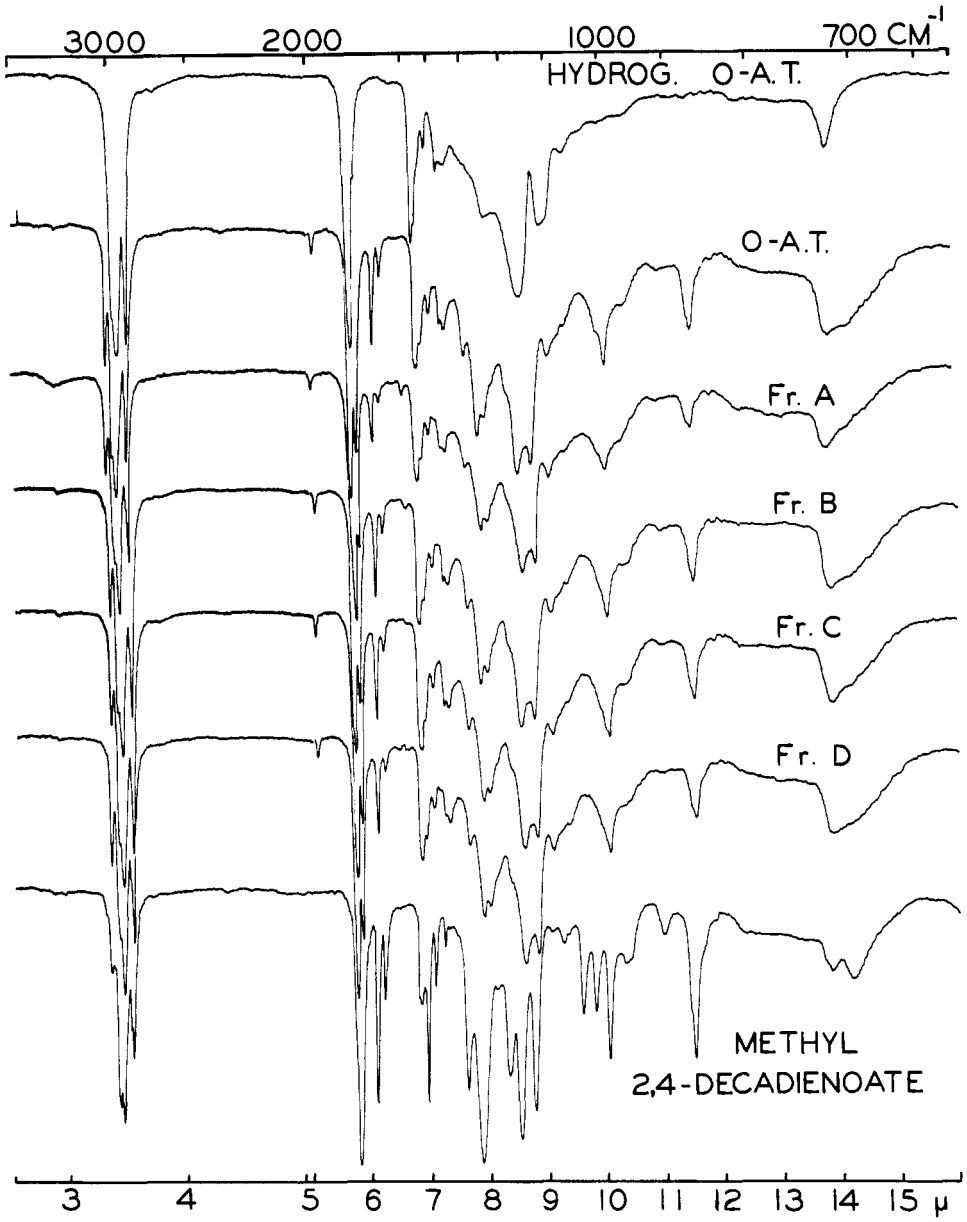
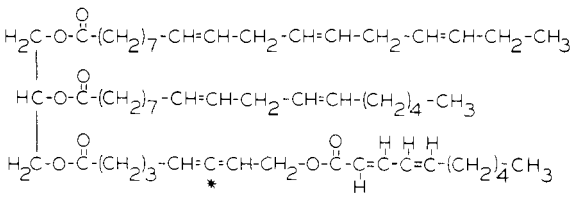


FIGURE 7: Infrared spectra of the hydrogenated tetraester triglyceride, the four countercurrent fractions of the optically active tetraester triglyceride, and methyl 2,4-decadienoate.

mass numbers greater at 277, 279, and 281 the pattern repeats, indicating acyloxy cleavage of the same components. The presence of a peak 14 mass units greater than acyloxy cleavage of linolenate, but not for linoleate or oleate, indicates that linolenate is on a primary hydroxyl group of the glycerol in the principal component of this fraction. Thus, the structural formula of the principal component is



The mass spectra of the other three countercurrent fractions showed that each was a mixture of two or more components. For each fraction, more than one significant parent peak was found. Before individual structures can be assigned to the triglycerides in any of the three fractions, further fractionation would be necessary to obtain the components in a more homogenous form.

Although the β-carbon of the glycerol moiety is a potential asymmetric center, naturally occurring triglycerides have not been isolated in an optically active form. This would suggest that, in nature, triglycerides occur either as racemic mixtures or that the long-chain acids on the two α-hydroxyls of glycerol do not provide a degree of asymmetry to the molecule sufficient to

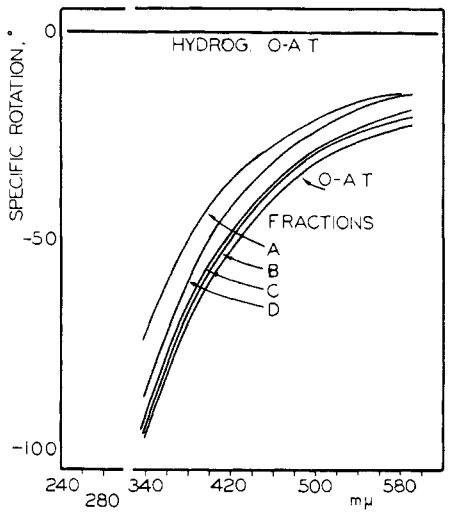


FIGURE 8: Optical rotatory curves of the optically active tetraester triglyceride, the four countercurrent fractions thereof, and the hydrogenated tetraester triglyceride.

rotate light to a measurable extent. The experimental evidence available supports the latter proposal. Long-chain diglycerides, synthesized stereospecifically, rotate light to a small but measurable extent (Baer and Buchnea, 1958), but short-chain diglycerides have a stronger rotation (Baer and Mahadevan, 1959). In contrast, triglycerides prepared in a stereospecific manner are optically inactive (Baer and Fischer, 1939; Schlenk, 1962) if the fatty acids are common fatty acids. However, Morris (1965) has synthesized asymmetric dipalmitin sorbate and found it to be optically active. In the biosynthesis of triglycerides and phospholipids in animal tissue, diglycerides serve as a common intermediate (Kennedy and Weiss, 1956; Weiss and Kennedy, 1956). Although the resulting triglycerides are optically inactive, the phospholipids with their greater degree of asymmetry rotate light to a significant degree.

Failure of the hydrogenated tetraester triglyceride to rotate light does not eliminate the β-carbon of glycerol as an asymmetric center. In the hydrogenated form, the only difference in the groups on the two primary glycerol hydroxyls is the internal ester function in the diester moiety. Because the internal ester is so far removed from the β-glycerol carbon, it would be expected to have little, if any, effect on the asymmetric properties of the molecule. In contrast, the β-carbon of the glycerol moiety in the native unsaturated triglyceride might be expected to contribute to the observed rotation because the difference in the moieties on the two glycerol α-carbons is very striking. Unfortunately, the optical activity, if any, contributed solely by the β-carbon of glycerol, is difficult to verify experimentally. This could be accomplished only if the allene function could be rendered racemic without racemizing the β-carbon of the glycerol.

Our failure to detect the allene-containing component in the previous study can be attributed to several factors. When the native optically active triglyceride was interesterified with methanolic HCl and the resulting methyl esters chromatographed on gas-liquid chromatography, there was no evidence of an unusual component. The structure of the allene component formed on interesterification would be the allylic alcohol, methyl 8-hydroxy-5,6-octadienoate. Because of the high reactivity of allylic alcohols, this compound may well have polymerized or undergone decomposition on the gas-liquid chromatographic column. In the previous study the $LiAlH_4$-acetylation technique had been used primarily as a means to identify glycerol. When the hydrogenated triglyceride was treated in this manner and the alcohol acetates separated by gas-liquid chromatography, a peak was observed with a retention time

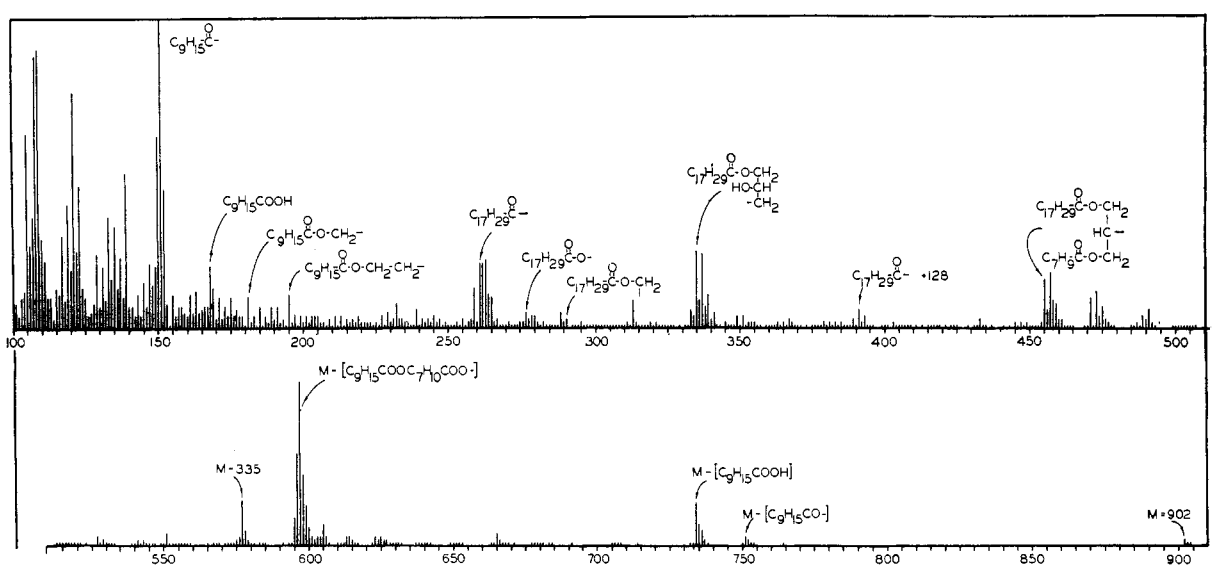


FIGURE 9: Mass spectrum of countercurrent fraction A.

identical with authentic glycerol triacetate. By coincidence, under the gas-liquid chromatographic conditions employed, 1,8-octanediol diacetate has a retention time identical with glycerol triacetate. When the native unsaturated triglyceride was treated in the same manner the resulting octadiene-1,8-diol diacetate appeared as a shoulder on the peak corresponding to oleyl acetate. Under any but ideal gas-liquid chromatographic conditions these two components appear as a single entity. In the previously reported study this peak was considered to be solely oleyl acetate.

Failure to detect the allene-containing component may be attributed, at least in part, to the lack of information about this type of compound. Although several antibiotics, such as mycomycin (Celmer *et al.*, 1952) and nemotin (Bu'Lock *et al.*, 1955) and a carotenoid, fucoxanthin (Torto and Weedon, 1955), contain an allene group, this function is uncommon in nature and not found hitherto in lipids. In the antibiotic field the allene group occurs in conjugation with other unsaturation and thus gives rise to a characteristic ultraviolet spectrum. The triglyceride isolated from the seed oil of *Sapium sebiferum* would, therefore, appear to be a new type of structure in two different respects. The allene function is an isolated function and is not conjugated with any other unsaturation. Instead it is located allylic to the hydroxyl of an ester linkage. Second, the tetraester triglyceride is the first triglyceride to be characterized containing four ester linkages. The only structural features of the molecule remaining unresolved are the stereoconfigurations surrounding the β-carbon of the glycerol moiety and the allene function.

Our first approach to elucidate the structure of the tetraester triglyceride involved lipase studies. Pancreatic lipase, under carefully controlled conditions, cleaves preferentially the fatty acid moieties esterified to the two primary hydroxyls of glycerol (Desnuelle and Savary, 1963). When this method of analysis was used on the tetraester triglycerides, the results obtained were confusing, but we could conclude that 2,4-decadienoic acid was present in a primary position. The work of Barber *et al.* (1964), describing the determination of triglyceride structure by mass spectrometry, suggests that this approach offers considerable advantage over lipase treatment. Once mass spectrometry was employed, the elucidation of the structure of the tetraester triglyceride proceeded in a straightforward logical manner. In order for mass spectral analysis to be of use in studies of glyceride structure the triglyceride must be obtained in a high degree of purity. Because naturally occurring triglycerides are a mixture of numerous closely related molecular species, the fractionation into single molecular entities is a formidable problem. Through the use of modern fractionation methods considerable progress has already been made in the area. Future work in our laboratory will involve the extensive use of mass spectrometry for the determination of the structures of naturally occurring triglycerides.

Acknowledgments

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